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The abuse of heroin (diamorphine) and heroin deaths are growing around the world. The interpretation of the toxicological results from suspected heroin deaths is notoriously difficult especially in cases where there may be limited samples. In order to help forensic practitioners with heroin interpretation we determined the concentration of morphine (M), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in blood (femoral and cardiac), brain (thalamus), liver (deep right lobe), bone marrow (sternum), skeletal muscle (psoas) and vitreous humor in 44 heroin related deaths. The presence of 6-monoacetylmorphine (6-MAM) in any of the postmortem samples was used as confirmation of heroin use. Quantitation was carried out using a validated LC-MS/MS method with solid phase extraction. We also determined the presence of papaverine, noscapine and codeine in the samples, substances often found in illicit heroin and that may help determine illicit heroin use. The results of this study show that vitreous is the best sample to detect 6-MAM (100% of cases), and thus heroin use. The results of the M, M3G and M6G quantitation in this study allow a degree of interpretation when samples are limited. However in some cases it may not be possible to determine heroin/morphine use as in 4 cases in muscle (3 cases in bone marrow) no morphine, morphine-3-glucuronide or morphine-6-glucuronide was detected, even though they were detected in other case samples. As always postmortem cases of suspected morphine/heroin intoxication should be interpreted with care and with as much case knowledge as possible.

1. Introduction

Heroin (diacetylmorphine or diamorphine (BAN)) is an opioid analgesic that is abused by around 16.5 million people worldwide and in 2015 was responsible for an estimated 150,000 deaths [1]. The use of heroin is however changing around the world. Europe is currently seeing a recent increase in deaths after seeing a long term decline in the use of heroin [2], this increase in abuse is also being seen in other parts of the world such as South West and Central Asia [1]. North America in particular has seen a large 145% increase in heroin users since 2007 [1]. Although there has been a recent increase in the use and abuse of synthetic opioids worldwide [2] the abuse of heroin is still a global problem to be addressed. Heroin is commonly abused by various routes the most common ones being intravenously, orally and inhalation (smoking) [3]. Heroin, once absorbed, is rapidly deacetylated and pharmacologically activated by metabolism with the formation of 6-monoacetylmorphine (6-MAM) with a half-life of about 2-5 minutes [4]. 6-MAM is then hydrolysed rapidly to morphine (t½ ~15 min). The major final steps of the metabolism of morphine are the conjugation of morphine to form the pharmacologically inactive morphine-3-glucuronide (M3G) and the pharmacologically active morphine-6-glucuronide (M6G). N-demethylation of morphine also results in the formation of the inactive minor metabolite normorphine [5]. Due to various factors it is difficult to interpret postmortem morphine concentrations. These factors include overlapping concentrations of morphine measured in deceased and living patients[6], the rapid development of tolerance to opiates in users [6] and the possibility of changes in the blood concentration of opiates after death [7]. Due to the routine introduction of LC-MS/MS into laboratories it is becoming more common to quantitate the concentrations of morphine, morphine-3-glucuronide and morphine-6-glucuronide in post mortem samples [8, 9] rather than to determine the concentration of free morphine and total morphine levels (free + conjugated morphine levels) to determine possible toxicity [10]. To fulfill the lack of information and help colleagues in data interpretation we report the concentration of morphine, morphine-3-glucuronide and morphine-6-glucuronide whilst also showing qualitative results for noscapine, papaverine and codeine in 44 cases in a series of commonly sampled postmortem matrices (femoral blood, cardiac blood, brain (thalamus), liver (deep right lobe), bone marrow (sternum), muscle (psoas) and (vitreous) using a validated LC-MS/MS method.

2. Material and Methods

2.1 Sample collection

All samples were collected and analysed as part of the routine autopsy procedure for suspected heroin deaths (2010-2012). These samples included femoral venous blood (unpreserved sample and preserved sample with potassium fluoride/sodium oxalate), unpreserved cardiac blood, muscle (psoas), bone marrow (sternum), liver, brain (thalamus) and preserved vitreous humour. Sample collection was performed according to the following protocol:

All fluid samples (blood, vitreous) were collected using a 12 gauge needle and syringe. Unpreserved fluid samples were stored in a 20 ml Sterlin® tube. Preserved samples were stored in a 2.5% w/v sodium fluoride/potassium oxalate tube. Femoral venous blood was collected by the superior clamping of the femoral vein before sampling. Cardiac blood was sampled for either of the ventricles. Vitreous humour was sampled from both eyes and mixed. For all tissue samples the area was wiped clean before sampling commenced, samples were stored in 20 ml Sterlin® tubes. For psoas muscle sampling an incision was then made anteriorly to remove approximately $1cm^3$ of interior tissue to minimise external contamination. For liver an incision was made across the middle right lobe to provide a $1cm^3$ of centrally located tissue to avoid external contamination. For bone marrow the sternum was cut laterally between the 1^{st} and 2^{nd} rib to allow access to the bone marrow. Tweezers were used to collect between 0.5 - 1 g of bone marrow from the sternum cavity. For the brain approximately 1 cm^3 of the thalamus was wiped clean then collected. Samples were stored at 4ºC for 1 week and then stored at -20 ºC until analysis. All analysis was completed with 5 weeks of the samples being collected.

2.3 Standard Solutions, Calibration Curve and Quality Control

Morphine (1mg/ml), morphine-3β-D-glucuronide (M3G) (1mg/ml) and morphine-6-β-D-glucuronide (M6G) (100µg/ml) methanolic standards (LGC Standards, Teddington UK) were used to prepare a fresh 25 mg/L combined standard solution then diluted with blank equine plasma (TCS Biosciences, Buckingham, UK) to prepare a 7 point calibration curve (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/L). Separate quality control (QC) standards were prepared in blank equine plasma at a concentration of 0.025 and 0.5 mg/L. Morphine-3β-D-glucuronide-D3 (M3Gd3) and morphine-D3 (Md3) both 1 mg/ml methanolic solutions (LGC Standards, Teddington UK) were diluted in water at the final concentration of 1mg/L and used as Internal Standards. HPLC grade acetonitrile, ammonium carbonate, ammonium formate, formic acid and HPLC grade methanol were supplied by Fisher Scientific (Loughborough UK).

2.3 Sample Preparation and extraction

2.3.1 Tissue Sample Preparation

For extraction, liver, brain, bone marrow and muscle were homogenised, adapting and applying a previous published procedure[11]. In summary tissues were dried using tissue paper, accurately weighted and homogenised (Janke & Kunkel Ultra-Turrax T25) with 4 parts of water. Thalamus and bone marrow were accurately weighted and homogenised with 2 parts of methanol and 6 parts of hexane. The methanol/hexane homogenates were then centrifuged and the top hexane layer discarded.

2.3.2 Sample Extraction

The sample extraction was based on a previously published method[10]. Following homogenisation 200 µl of liver was diluted with 1000 µl equine plasma. For all the other biological samples, quality controls and calibrators, 150 µl of sample was diluted with 150 µl equine plasma. 900 µl of liver sample and 300 µl of all the other biological samples were spiked with 50 µl of Md3 and 50 µl of M3Gd3 (IS). After vortexing, 1ml of 0.5M Ammonium Carbonate solution was added to each sample, now ready to be extracted. Solid phase extraction was performed using a Varian Bond Elut LRC-C_{18}, 200mg cartridges. The

cartridges were conditioned using 2ml of methanol followed by 2ml of water and finally 1ml of 0.5M ammonium carbonate solution. Then, 1ml of the sample was loaded onto the cartridge and allowed to drain to the clear tube. The cartridges were washed using 5ml of 0.005M ammonium carbonate solution the washing solution discarded and the cartridges dried for 5 minutes. 1ml of 70:30 ACN:H_2O solution was loaded onto the cartridges and allowed to elute into a clean glass vial. The eluents were evaporated to dryness under air at 45°C. The evaporated eluent was reconstituted with 100 µl of freshly made LC-MS mobile phase (96% phase A: 4% phase B), transferred into a HPLC vial for analysis.

2.4 Sample Analysis

A 1200 Agilent HPLC system interfaced with an Applied Biosystem 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer (AB Applied Biosystem, Foster City, CA, USA) equipped with an electrospray ionization source (ESI) was used to analyse all the samples. Data acquisition was achieved with Analyst 1.5 software. Separation of all analytes was accomplished using a Phenomenex Synergi 4µ Polar-RP 80A Column (150 mm × 2 mm × 4µm) protected by a Phenomenex Security Guard column (Macclesfield, UK). Mobile Phase A consisted of a 1mM ammonium formate and 0.1% formic acid solution (around pH 2.7). Mobile Phase B was a 50% acetonitrile solution with 1mM ammonium formate and 0.1% formic acid (around pH 3.8).

The qualitative and quantitative screening were based on a previously published method [10]. For the qualitative screening a gradient method was applied. Following injection the eluent was at 1.5% ACN (97% A, 3%B) for the first 3 min, ramping to 47.5% ACN over 5 min (5% A, 95%B) and held for 3.5 min. The eluent was then reduced back to the initial 1.5% ACN over 0.5 minutes and was held for a further 3 minutes. This gave a total run time of 15 minutes. Flow rate was 0.6 ml/min, injection time was 20 seconds and column temperature was set at 40 °C. The injection volume was 20µl. The following ion transitions and retention times were observed: Morphine (*m/z* 286/286, 286/201, 286/165), morphine-3-glucuronide (*m/z* 462/462, 486/286, 462/268), morphine-6-glucuronide (*m/z* 462/462, 486/286, 462/268), 6-monoacetylmorphine (6-MAM) (*m/z* 328/328, 328/193, 328/165), noscapine (*m/z* 414/414, 414/220), papaverine (*m/z* 340/340, 340/202), codeine (*m/z* 300/300, 300/215, 300/165). Internal standards: Morphine-d3 (*m/z* 289/289, 289/201, 289/165), morphine-3-glucuronide-d3 (*m/z* 465/465, 485/289, 465/204).

Free morphine, M3G and M6G quantitation was achieved with an isocratic method of 1.5% acetonitrile (97% A, 3%B). The triple-quadrupole linear ion trap mass spectrometer performed the scan in positive mode using ion spray ionization (voltage 5500 V). The ion source temperature was set at 700 °C, DP 20 V, EP 10 V.

2.5 Method Validation

The method was validated according to the guidelines of Peters *et al.* [12], with the matrix effects being evaluated by the methods of Matuszewski *et al.* [13]. The morphine, morphine-3-glucuronide and morphine-6-glucuronide linearity (r^2 >0.99) was achieved applying a linear regression with a 1/x weighting factor on a seven points calibration curve (0.01 mg/L, 0.025 mg/L, 0.05 mg/L, 0.1 mg/L, 0.25 mg/L, 0.5 mg/L and 1 mg/L). A series of quality control samples in plasma at concentration of 0.025 mg/L and 0.5 mg/L for morphine and morphine glucuronides were prepared and analysed on different days to determine the precision (CV%) and accuracy (bias) of the method. Accuracy and bias (both interday (n=5) and intraday (n=30) were within the acceptable ranges (±15%) proposed by Peters *et al.* [12]. The MRM transitions selected for quantitation were the following: for morphine 286/165 (against Morphine-d3 289/152), for Mophine-3-and-6-glucuronide 462/286 (both against Morphine-3-glucuronide-d3 465/289). Lower limit of quantification and (LLOQ) and limit of detection (LOD) were determined as the minimum concentration that would produce a signal-to-noise ratio of ten and three respectively. The LOD for morphine, morphine-3-glucuronide and morphine-6-glucuronide were 0.004 mg/L, 0.003 mg/L and 0.004 mg/L respectively, with the LLOQ being 0.01 mg/L for all compounds. Evaluation of the results obtained for morphine, morphine-3-glucuronide and morphine-6-glucuronide in all biological matrices analysed indicated that it was possible to use the calibration curve in plasma for quantitation since all spiked tissue concentrations were all within 20% of the expected concentration.

To investigate matrix effects; morphine, morphine-3-glucuronide and morphine-6-glucuronide were spiked (along with IS) at different concentrations (0.025 mg/L and 0.5 mg/L) in at least 5 different samples of SAGM blood (Transfusion Unit. Ninewells Hospital, Dundee, UK), porcine vitreous, homogenised porcine/bovine bone marrow, bovine muscle, porcine brain and porcine/bovine liver (all local butcher); extracted, analysed and measured

against the plasma calibration curve to determine the deviation from the true value. Evaluation of the results obtained for morphine, morphine-3-glucuronide and morphine-6-glucuronide in all biological matrices analysed indicated that it was possible to use the calibration curve in plasma for quantitation since all spiked tissue concentrations were all within 20% of the expected concentration. The concentrations of the solid matrices were calculated according to the method of Flanagan *et al.* [11]. The validation results are shown in table 1.

2.6 Statistical Analysis

Morphine results obtained from all biological matrices underwent statistical Pearson Correlation analysis to determine whether any linear relation exist between different samples. The data was also analysed using "Principal Component Analysis" (PCA).

PCA is an unsupervised multivariate procedure and is a well-known linear data compression and feature extraction technique [14]. It derives new, uncorrelated variables that are linear combinations of the original variable set ordered by reducing variability. PCA is mainly used to reduce the dimensionality of a data set while retaining as much information as possible by eliminating the lowest-ranking variables. It is a simple and fast method but remains a linear approach, so any nonlinear correlation between variables will not be retained. The scores produced may be plotted in two or three dimensions to inspect the data. Therefore it might be possible to relate the data using statistical methods such as principal component analysis (PCA).

3. Results and Discussion

A total of 44 cases of death from heroin use were investigated. The presence of 6-monoacetylmorphine (6-MAM) in at least one of the case samples and the case circumstances were used as confirmation of illicit rather than medicinal heroin use. Each sample collected at autopsy (cardiac blood, femoral blood, preserved (fluoride/oxalate) femoral blood, preserved vitreous, liver, bone marrow, psoas muscle and brain) underwent initial qualitative screening for morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, 6-MAM,

papaverine and noscapine). Following the quantitative screen the levels of free morphine, morphine-3-glucuronide and morphine-6-glucuronide were determined in all samples.

3.3 Age and gender of heroin users

Of the 44 cases of death following heroin use 79.5% were male with a mean age of 36 years; (median 35 years; range 21 to 65 years). 20.5% were female with a mean age of 34 years, (median 33 years; range 21 to 46 years). The mean and median age for all cases (regardless of sex) was 35 years. Averages for age and gender were similar to previous other studies of heroin related fatalities [6, 15]

3.1 Qualitative Screening

In the case of decomposition the full range of toxicological samples may not be available for testing. In order to determine the samples in which morphine, morphine-3-glucuronide and morphine-6-glucuronide can be detected we carried out qualitative screening of blood (femoral and cardiac), vitreous, liver, brain (thalamus), muscle (psoas) and bone marrow. The results of the qualitative screening are found in Table 2. The presence of morphine was confirmed in all cases for cardiac blood, unpreserved femoral blood, preserved femoral blood and vitreous with high levels of detection of morphine and in brain, liver, bone marrow and muscle samples (98%, 95%, 91% and 86% detection) respectively. Similar to the study by Thaulow *et al* [16], morphine-3-glucuronide was detected in all case samples for cardiac blood and unpreserved femoral blood. High levels of detection were observed in all other matrices; preserved femoral blood (98%), brain (84%), liver (98%) bone marrow (75%) samples, vitreous (95%) and muscle (84%) samples. Previous work [16] has only determined detection of M3G in vitreous (88%) and muscle (89%) which are similar to our data. Thaulow *et al.* [16] in agreement with our data detected morphine-6-glucuronide in fewer matrices than morphine and morphine-3-glucuronide, in our data M6G exhibited with high rates of detection >86% in all samples apart from muscle (50%) bone marrow (41%) and thalamus (43%). This would be expected as more M3G is produced by metabolism of morphine than M6G [17]. However we detected M6G in psoas in a higher number of cases (50% vs 9%) and also in vitreous humour (84% vs 44%) than the Thaulow *et al.* study [16]. These results suggest that the best matrices for the confirmation of free morphine and its glucuronide metabolites are blood (femoral and cardiac) > liver > vitreous. In muscle, bone marrow and brain, morphine will be detected in

most cases but not all of the time. This may mean that in decomposed bodies the use of morphine may not be able to be confirmed even if the deceased had taken morphine. The presence of morphine and its main metabolites morphine-3-glucuronide and morphine-6-glucuronide have previously been reported in a number of human cases for various matrices and give similar results to those that we found in this study with morphine-3-glucuronide and morphine-6-glucuronide detectable in blood, vitreous, brain, liver, lung and kidney [18-24].

The presence of morphine alone does not confirm the use of heroin as morphine can be produced as a metabolite of codeine and morphine can be abused by itself. In order to definitively identify the use of heroin, 6-MAM needs to be detected. However 6-MAM is unstable in the body being metabolised even after death by cholinesterases. With particularly high concentrations of esterases found in liver, heart, lung, kidney and blood [25]. Our study shows that as with other studies [24, 26, 27] vitreous is the best sample for the detection of 6-MAM being detected in 100 % of cases. In blood the 6-MAM was most stable in fluoride/oxalate preserved samples where it was detected in 61% of samples. In all other matrices 6-MAM was detected in <52% of case the worst samples for the detection of 6-MAM was liver and bone marrow (2% and 5% respectively). However it is unclear why so little 6-MAM was detected in bone marrow as in previous human and animal studies 6-MAM has been shown to be detectable 2 months after death [23, 28]. This may be due to the different sampling sites used between this study (sternum) and the previous studies (both femur) or a lack of time for 6-MAM to equilibrate with the bone marrow compartments before death occurred.

Another important piece of information that can be obtained from toxicological screening is the determination of the use of heroin which can come from either illicit or medicinal (diamorphine) sources. The illicit heroin contaminants which occur in the largest amounts due to the processing of the opium poppy are noscapine, papaverine, codeine, 6-acetylcodeine, thebaine, and narceine [29]. The most commonly used biomarkers for illicit heroin are noscapine and papaverine as they are found in the opium poppy and have been found to survive the common two methods of heroin production, the lime method (South East Asia) and the ammonia method (West Asia) [30]. The amount of noscapine and papaverine has been found to vary based on the batches of heroin. In 513 illicit seizures of heroin from the

Netherlands between 1994-1999 noscapine was detected in 15% and papaverine detected in 1.3% (median values) [29]. In 85 Iranian heroin samples (2008-2009) Akhgari *et al* [31] reported that 43.5% of samples contained noscapine and 42.4% papaverine. From another study carried out by O'Neil *et al* [32] only 5% of samples of Southeast Asian Origin contained papaverine and noscapine, where none of the illicit heroin of Indian origin had either papaverine or noscapine.

It was also reported that 59% of illicit heroin of Pakistani origin contained noscapine and papaverine, whereas 88.4 % and 88.1 % of Iranian and Turkish origin respectively contained noscapine and papaverine. Illicit heroin samples have also been found not to contain noscapine and papaverine [29-32] so with these studies showing that varying concentrations of noscapine and papaverine can be found in illicit heroin samples and as both noscapine and papaverine can be found in over the counter drugs and foods containing poppy seeds. The centrally acting cough suppressant noscapine is commonly prescribed in Asia and several European countries and papaverine is available for the treatment of erectile dysfunction and as an analgesic as a component of papaveretum which contains morphine, papaverine and codeine [33]. Several studies show that noscapine and papaverine are not present in illicit heroin samples and therefore negative results do not infer that illicit heroin was used. Use of illicit heroin attributed due to the presence of noscapine and papaverine should be done after excluding their provenance from other prescribed drugs and from patients that were given atracurium.

Overall for all compounds investigated we found a slightly higher percentage of positive cases for preserved matrices (blood and vitreous) showing that more information can be gained from preserved blood samples compared to unpreserved blood samples.

3.2 Quantitative results:

Interpretation of the postmortem results requires investigation (case history), pathology and also laboratory analysis that are evaluated in the context of each other [34]. In order for laboratory investigations to be placed into correct context data must be obtained from case studies of heroin death. To the best of the authors' knowledge this is the first publication that has complete case studies for heroin deaths in a variety of matrices with both free morphine

and its glucuronides. We determined the concentrations of free morphine, morphine-3-glucuronide and morphine-6-glucuronide in a variety of matrices as shown in Table 3. An overall summary of the results are given in table 4. The general consensus is that 'gold standard' sample for interpretation in postmortem toxicology is that of femoral blood which is thought to be affected the least by the "toxicological nightmare" of postmortem drug redistribution [7]. The free morphine levels in unpreserved femoral blood (median 0.21 mg/L, range 0.01-1.35mg/L, n=44) were not found to be significantly higher than that found in preserved femoral blood (median 0.14 mg/ L, range 0.01-1.19 mg/L, n=44). As can be seen in table 5 the mean values of free morphine are similar to a variety of other studies that reported free morphine levels in femoral blood. These results overlap with the free morphine blood levels reported in apprehended drivers in Sweden median 0.15 mg/L range 0.02-1.13mg/L. n=124 [6] and so confirm other reports that the confirmation of death due to morphine must be confirmed from as much case information as possible rather than looking at drug reference ranges [35]. The morphine results that have typically been used in the past to determine the role of morphine in opiate deaths has been that of free morphine (unmetabolised morphine) and total morphine (unmetabolised morphine and metabolised morphine). However this does not allow investigation of the individual roles of the glucuronide metabolites in opiate toxicity. Due to the lack of previous studies, especially in non-blood matrices studies we also investigated the levels of morphine-3-glucuronide and morphine-6-glucuronide. The results are shown in table 2 and 3 as shown in table 5 these results are similar to the ranges that have been reported in previous studies that measured femoral blood. It has been suggested that the molar ratio of M3G:M6G might be responsible for the analgesic effect (and thus toxic effects of morphine) [36] however this is based on the assumption that M3G antagonises the analgesic (and also respiratory depressant) effects of M and M6G. This effect has been demonstrated *in vivo* following intracerebroventricular injections [37, 38] but has not been repeated in either animal studies [39, 40] or human studies in the more physiologically relevant intravenous injection [41]. So the M3G:M6G ratio is unlikely to indicate the effects of morphine as previously suggested. Its relevance has also been called into question as we would have to assume that no detectable levels of both M3G and M6G were present before the administration of heroin, something that may not be true in chronic heroin/morphine users. It has also been found that the metabolites are not produced in equal amounts and that the amounts of glucuronide metabolites can depend on the route of administration [17].

Morphine-6-glucuronide has been shown in numerous studies to have a similar or greater analgesic potency than morphine [41-43], like morphine it is also thought to give a similar level of reward as morphine [44-46] but appears to have less of a respiratory depressant effect than morphine in human studies [47] possibly due having less affinity for the μ2 opiate receptor than morphine. The μ2 opiate receptor is thought to be responsible for the side effects of opiate drugs. Both morphine and M6G have similar affinities for the μ1 receptor [48, 49].

In all cases the concentrations of free morphine, morphine-3-glucuronide and morphine-6-glucuronide were higher in cardiac blood than femoral blood. This was as expected as in previous studies that investigated both cardiac and femoral blood the cardiac blood was generally found to be higher than the femoral blood [50-52]. Two studies have shown concentrations of free morphine that have been higher in femoral blood than cardiac blood. In one case the cardiac morphine concentration was 0.8mg/L (left ventricle), 0.46mg/L (right ventricle) with a femoral blood of 1.35mg/L [20]. In a study performed on 4 cases of heroin related deaths Skopp analysed the concentration of morphine, M3G and M6G sampling the blood from different sites: aorta, infra- and suprarenal potion of inferior vena cava, the superior vena cava, the femoral and subclavian vein, right and left ventricles [53]. Case number one showed concentration of cardiac blood (left and right ventricle) for all three compounds higher than the one in femoral blood (left and right femoral vein). For the second case morphine concentration in the blood from the right ventricle was lower than the blood in the right femoral vein. In the same case M6G was overall higher in cardiac blood than femoral blood. M3G was not quantifiable in cardiac blood due to biological matrix interferences. The third case morphine in left femoral blood was lower than the left ventricle but higher than the right ventricle, and also M6G presented a higher concentration in the blood from the left femoral vein than the blood of the left ventricle. Case four had a lower concentration for M3G and M6G in the blood from the left ventricle than the right and left femoral vein.

Liver was historically a common tissue for toxicological analysis due to its large mass. We also investigated the free morphine & morphine glucuronide metabolites in the right lobe of the liver a part of the liver that is thought to be the least effected by postmortem redistribution, especially from the stomach [7]. Concentrations of free morphine in liver

showed a mean concentration of 0.62mg/kg with a range of 0-2.55mg/kg, M3G was 0.7mg/kg (range 0-4.68), M6G was 0.29mg/kg (range 0-1.76). We are the first study to show free morphine and the glucuronide metabolites of morphine in liver samples. Only Klingman reported in study of one case (intoxication following morphine, tramadol and atracurium intake) the concentration of morphine as well M3G and M6G in liver which were 2.32, 14.94 and 15.1 mg/kg respectively [55]. Three other studies investigated free morphine concentrations alone. Wyman analysed 25 heroin deaths. Morphine concentration in liver averaged 0.04-1.56 mg/kg (mean 0.33 mg/g) so slightly lower than our results [18]. Moriya described just one case with a morphine concentration of 1.44 mg/kg [20]. Felby investigated morphine concentration in the liver of 14 heroin deaths. The range reported was 0.4-18 mg/kg, average 2.57mg/kg [21]. In our study liver was the biological matrix showing the highest mean concentration of morphine (0.62 mg/kg). In the other studies investigating liver they also found liver to contain the highest concentration of morphine of all the matrices tested [18, 20, 21, 54]. The high concentrations of morphine and metabolites in the liver may mean that the liver is a possible depot for postmortem redistribution.

Muscle is commonly used for detection of morphine use rather than for interpretive purposes [55]. However it has been suggested that muscle may give a reasonable estimate of the concentration of a drug in the body at the time of death [56]. We quantitated free morphine, M3G and M6G in the psoas muscle. Results given as mean and range. Morphine 0.19mg/kg, 0-1.13mg/kg; M3G 0.09mg/kg, 0-0.91mg/kg; M6G 0.03mg/kg, 0-0.15mg/kg. As with the studies in the liver previous studies have only investigated free morphine concentrations in the muscle and didn't investigate M3G and M6G. The other studies, muscle was either from a different site (thigh) or was not given which as drug concentrations have been shown to differ in the muscle depending on the site of sampling [57] the results may not be directly comparable to the current study. With Moriya, 1 case of heroin death was considered. Right femoral muscle had a morphine concentration of 0.74 mg/kg [20]. Felby reported the results of morphine concentration analysed in 14 cases of victims due to heroin intake [21]. Muscle morphine concentration averaged 0.39 mg/kg (range 0-2 mg/kg). Morphine concentration in muscle was 1.13 mg/kg in Klingmann study [54]. All of these study demonstrated larger concentrations of morphine compared to the data presented here.

Bone marrow has been suggested as a possible source of information about morphine in decomposed bodies due to bone marrow being protected from the environment in decomposition situations [23]. We found that the mean concentration of free morphine in bone marrow was 0.25 mg/kg range 0-3.23 mg/kg. This was similar to the bone marrow morphine level found in a heroin addict immediately after death (0.2 mg/kg). It is unclear if the concentrations of morphine vary depending on the bone marrow sampling site.

The brain is an important site of action for the respiratory depressant effects of morphine, the effect that is thought to cause death in heroin fatalities [58]. We investigated the concentration of free morphine in the thalamus. The mean concentration of free morphine was 0.26 mg/kg (range 0-1.96 mg/kg); M3G 0.06mg/kg, 0-1.12 mg/kg; M6G 0.01 mg/kg. Previous studies have looked at the concentrations of morphine in the brain, but as with other tissues there is no standard sampling site. One study looked at two regions in the brain medulla (mean 0.24 mg/kg range 0.05-0.45mg/kg) and cerebellum (mean 0.34 mg/kg, range 0.05-0.67mg/kg) [59]. These results were similar to those that we obtained but may indicate that the concentration of morphine is not uniform thorough out the brain and for future comparison of cases specific sampling sites should be identified. Other cases where the brain was analysed also gave similar concentrations as found in our study. Moriya [20] presented the results of a subject who died after injecting heroin and methamphetamine. Cerebrum was analysed morphine concentration was 0.34 mg/kg. In another study [22] Pare analysed morphine concentration in the thalamus 0.24 mg/kg (range 0-0.67 mg/kg). Only one study investigated the concentration of morphine as well as its metabolites in and unidentified area the brain. Klingmann found the concentration of morphine was 0.52 mg/kg, M3G 1.42 mg/kg and M6G 1.24 mg/kg [54].

As discussed above vitreous is commonly used to confirm the use of heroin, via the detection of 6-MAM when morphine is detected due to the stability of 6-MAM in vitreous but is not commonly used for the quantitation of morphine levels. The median concentration of morphine, M3G and M6G in this study for vitreous were 0.08mg/L (range 0.01-0.49 mg/L); 0.10 mg/L (range 0.01-0.65 mg/L) and 0.02 mg/L (0.01-0.19 mg/L) respectively. The morphine concentration here reported was the lowest among all the matrices analysed. Other studies that quantitated morphine and its metabolites in vitreous have reported concentrations of morphine and its glucuronides with similar levels than us. Morphine median 0.05mg/L (range 0.006-0.195 mg/L); M3G median 0.16 mg/L (range 0.036-0.250 mg/L) and M6G

median 0.02 mg/L (0.003-0.046 mg/L) [19] Morphine median 0.06mg/L (range 0.011-0.46 mg/L); M3G median 0.12 mg/L (range 0.016-2.0 mg/L) and M6G median 0.051 mg/L (0.014-0.31 mg/L) [16] and showing that quantitation of morphine and its metabolites can be achieved.

3.3 Relationships between and femoral blood and the other various matrices

With the various states that bodies can be found in due to the manner of death or subsequent decomposition femoral blood may not be available. In the past it has been suggested that other biological samples may be used to predict the femoral blood sample for subsequent analysis [60]. For this reason we investigated the correlation of femoral blood with the various matrices. For free morphine cardiac blood (R^2 = 0.79), bone marrow (R^2 = 0.70), muscle (R^2 = 0.69) and brain (R^2 = 0.73) significant correlations were observed, which were not seen with liver (R^2 = 0.49) and vitreous (R^2 = 0.44). These results in vitreous when compared to femoral blood are in agreement with a previous study (R^2 = 0.26; n=40 [61], as are the results of femoral blood to cardiac blood (R^2 = 0.76) and psoas muscle (R^2 = 0.75) [16] but don't agree with the results of two other studies that showed significant correlations between femoral blood and vitreous concentrations (R^2 = 0.87; p<0.01, n=69 [62]), (R^2 = 0.72; p<0.001, n=52 [60]) and (R^2 = 0.79; p>0.001, n=45 [16]). The differing sets of results obtained by the various groups and this study show that although correlations may exist for populations due to differences such as individual tolerance, individual metabolism, survival time after the administration of the drug and also postmortem redistribution [63, 64] that in the absence of a femoral blood sample in a case other samples should not be used to attempt to determine the femoral blood concentration.

Due to the complex nature of the data collected the visualisation techniques PCA was applied to the data, below in Fig 1 it is shown the 3D scatterplot of morphine results. The figure shows the PCA scores from the Morphine levels in different matrices represented by the first three PCs. PC1 contains 78.7 % while PC2 contains 8.1% and PC3 contains 5 % of the total variance. All matrices from different sites form three groups or classes in the plot: blood, bone marrow and brain at the bottom; muscle and liver at the top, vitreous by itself at the centre. The plot shows that most biological samples are visually separable when the first three PCs are utilised, with cardiac blood and brain showing highest correlation. However, a

prediction of result could not be extrapolated since the r square of all possible combination was not higher than 0.87 (cardiac blood and brain) (Table 6).

3.5.2 Results above 0.3mg/L for cardiac blood

In order to investigate whether our data corresponded to Logan's work [65] we also analysed data for morphine concentration in cardiac blood above and below 0.3mg/L.

In the PCA plot below (Fig. 2) PC1 contains 73.7 % while PC2 contains 10.6% and PC3 contains 6.7 % of the total variance. The situation represented in the plot was completely different from the previous ones, showing all matrices but vitreous grouped at the bottom of the plot, with bone marrow and brain overlaid, all the bloods close to each other, liver and muscle beside each other.

From the Pearson correlation table below brain and cardiac blood showed again to be the two matrices with the highest correlation. Cardiac blood and femoral blood r square was 0.7, cardiac blood and preserved femoral blood was 0.8 (Table 7).

3.5.3 Results below 0.3mg/L for cardiac blood

For cardiac blood results below 0.3 mg/L the PCA plot and the Pearson table (Fig. 3 and Table 7) showed a complete opposite situation compare to the one described by Logan. There was not significant correlation between cardiac and femoral blood with r square below 0.57.

3.6 Postmortem redistribution of morphine, morphine-3-glucuronide and morphine-6-glucuronide

Postmortem redistribution is the change in concentration of a drug (or chemical) at a specific anatomical site in the body in the time following death. This can result in the decrease, or most commonly increase in the concentration of a drug after death leading to complications in the interpretation of postmortem drug concentrations. A ratio of cardiac blood (CB) to peripheral (femoral) blood (FB) drug concentration >1 may indicate that a drug undergoes postmortem redistribution. The mean CB:FB ratio of morphine in the cases was 1.8 (range 0.1-11.0) similar to previous studies 1.2 (range 0.05-2.8) [66], 2.2 (range 1.0 – 5.8) [67] and 2.2

(range 0.2-9.2) [65]. Our mean CB:FB for M3G 2.7 (range 0.0 – 8.7) and M6G mean 2.6 (range 0.5 – 8.8) were larger than those previously reported 1.1 and 1.3 respectively [68]. Indicating that M, M3G and M6G may all undergo postmortem redistribution however this ratio may also however just represent anatomical site-to-site differences in drug concentration due to incomplete distribution before death [69] and more studies may be needed to confirm this.

3.5 Conclusions

The results of this study give 44 complete cases with morphine, M3G & M6G concentrations in a variety of common samples to aid practitioners in the interpretation of heroin and morphine related cases. Due to the possible postmortem redistribution of morphine and its metabolites the best sample to use for interpretation of the possible role of morphine in a death is femoral blood. Although some other sample morphine concentrations correlate well with those of femoral blood (bone marrow, muscle and brain) they should not be used to try to estimate the femoral blood concentration, but may be able to aid interpretation when blood samples are not available. Analytical determination of the use of heroin rather than morphine or codeine require the detection of 6-MAM, this work confirms, along with data from other studies that vitreous is the best sample to use, followed by blood and brain. Where blood is not available in some cases it may not be possible to determine heroin/morphine use as in 4 cases in muscle (3 cases in bone marrow) no morphine, morphine-3-glucuronide or morphine-6-glucuronide were detected, even though they were detected in other case samples. The best sample after blood for the detection of morphine was either the liver or the brain. The postmortem concentrations of morphine seen in these, and other postmortem cases overlap with seen in previously reported DUID cases. So as with all interpretations of postmortem drug concentration they should be carried out with as much case knowledge as possible.

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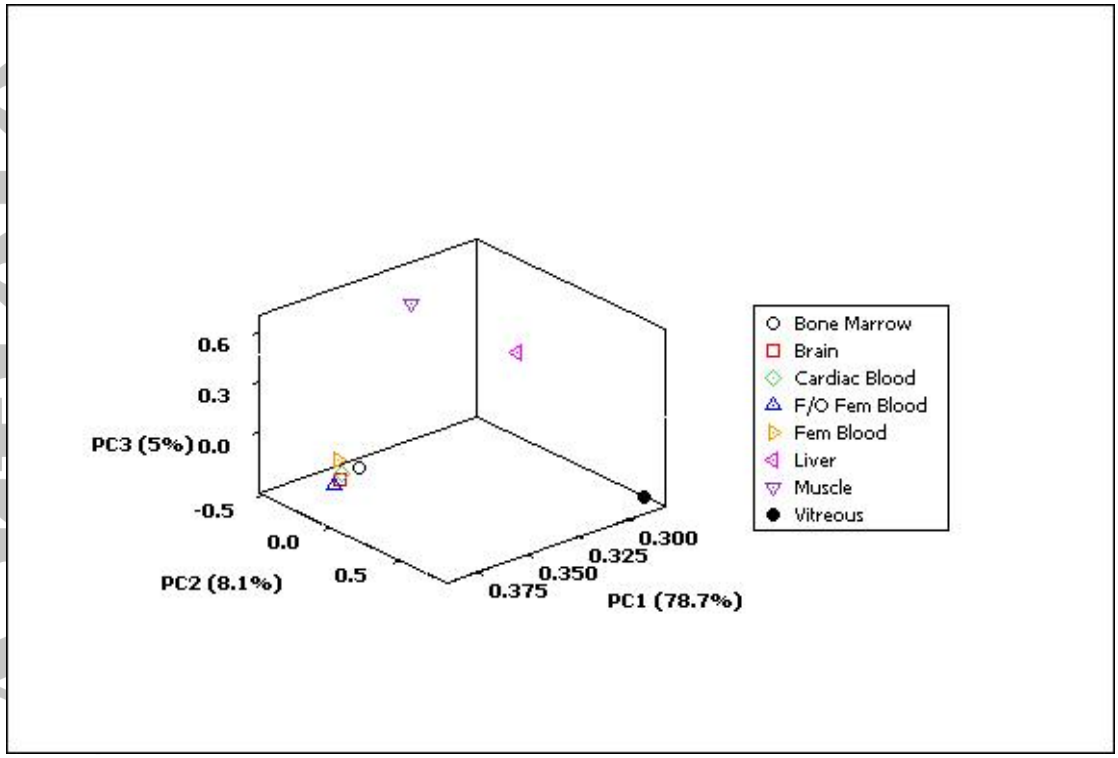

Fig 1: 3D scatterplot for morphine concentration for all biological matrices analysed

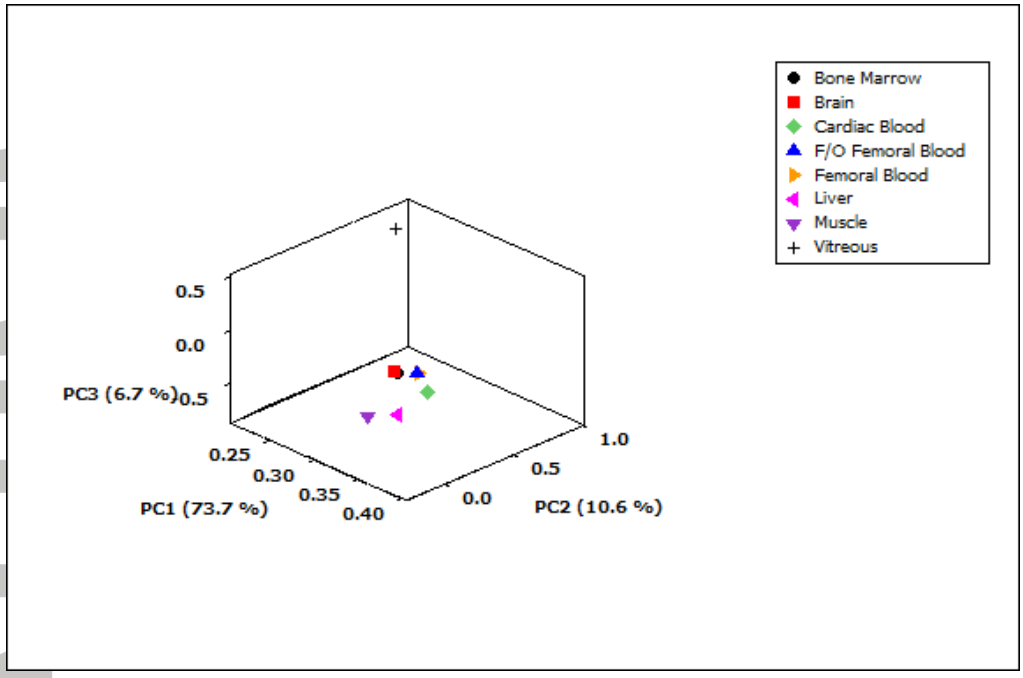

Fig 2: 3D scatterplot for mophine concentration with cardiac blood results higher than 0.3 mg/L

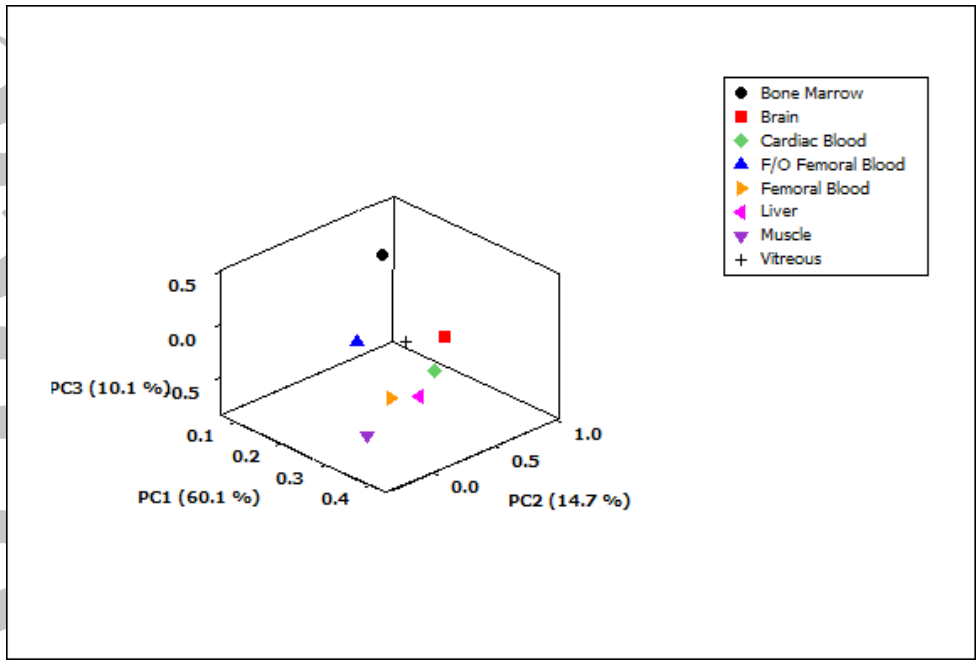

Fig 3: 3D scatterplot for morphine concentration with cardiac blood results below 0.3 mg/L

Table 1: A) Validation Data for Quantitation of Morphine in Various Matrices by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

	Plasma		***Blood***		***Liver***		***Muscle***		***Bone Marrow***		***Brain***		***Vitreous***	
QC Concentration (mg/L)	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**
Accuracy (%)	4.4	-0.2	0.8	-3.5	4.9	-8.9	6.6	-5.0	7.4	-14.6	8.6	8.2	12.2	-4.9
Precision (Within-run) (%CV)	9.5	7.6	2.7	1.9	2.1	1.9	1.3	1.97	3.1	1.6	11.4	3.5	3.58	0.9
Precision (Between-run) (%CV)	-12.9	14.8	6.1	6.5	8.9	3.1	8.6	11.2	3.7	8.4	8.1	5.1	11.8	3.6
Ion suppression/Enhancement (%)	-7.9	-11.0	-11.3	-7.7	2.0	-10.4	-8.2	-2.4	-15.3	-12.9	-12.5	5.3	5.0	-6.1
Recovery (%)	76.3	77.7	65.0	56.4	37.9	47.8	93.1	73.8	88.1	102.6	81.1	79.1	72.2	75.1

Table 1 B) Validation Data for Quantitation of morphine-3-glucuronide in Various Matrices by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

	Plasma		***Blood***		***Liver***		***Muscle***		***Bone Marrow***		***Brain***		***Vitreous***	
QC Concentration (mg/L)	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**
Accuracy (%)	1.9	0.6	6.9	7.25	14.1	14.2	-9.0	-2.3	-1.34	-7.7	5.0	6.3	9.51	14.82
Precision (Within-run) (%CV)	10.1	8.1	10.6	7.1	14.7	1.7	0.3	4.4	2.5	8.1	8.0	5.5	11.54	13.1
Precision (Between-run) (%CV)	-13.0	-10.6	11.6	9.6	12.2	11.8	7.9	9.7	12.3	11.3	13.7	5.6	9.9	7.5
Ion suppression/Enhancement (%)	19.3	-1.3	3.4	7.4	2.8	9.8	7.0	3.3	-3.5	-7.6	10.2	19.1	19.4	14.8
Recovery (%)	62.1	62.0	62.3	66.2	36.5	47.2	105.5	108.1	97.5	94.6	84.2	76.2	67.8	62.8

Table 1 C) Validation Data for Quantitation of morphine-6-glucuronide in Various Matrices by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

	Plasma		***Blood***		***Liver***		***Muscle***		***Bone Marrow***		***Brain***		***Vitreous***	
QC Concentration (mg/L)	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**	**0.025**	**0.5**
Accuracy (%)	6.4	6.3	10.2	12.6	12.7	-11.1	14.4	-14.5	3.1	-7.1	9.9	6.7	-9.12	13.5
Precision (Within-run) (%CV)	8.8	11.4	4.53	5.6	14.3	4.3	3.4	2.9	6.5	5.4	6.4	2.0	4.8	0.2
Precision (Between-run) (%CV)	-11.9	9.3	11.7	14.4	14.7	12.2	8.6	8.4	14.9	14.7	13.2	9.2	12.6	14.7
Ion suppression/Enhancement (%)	-9.4	-13.7	-0.5	-3.2	-17.2	-13.7	9.6	19.5	9.8	9.6	13.8	-1.9	-8.4	2.3
Recovery (%)	75.6	71.2	66.7	44.9	36.5	47.2	81.2	96.7	93.8	97.9	105.4	104.7	60.0	65.3

Table 2: Frequency of positive results for each compound (expressed as a percentage) among all 44 cases analysed.

	Morphine	M-3-G	M-6-G	6-MAM	Codeine	Noscapine	Papaverine
Cardiac Blood	**100**	**100**	**86**	**43**	**82**	**84**	**59**
Fem Blood	**100**	**100**	**86**	**52**	**77**	**82**	**50**
Liver	**95**	**98**	**89**	**2**	**55**	**75**	**59**
Muscle	**86**	**84**	**50**	**32**	**34**	**64**	**41**
Bone Marrow	**91**	**75**	**41**	**5**	**41**	**70**	**52**
Brain	**98**	**84**	**43**	**43**	**55**	**68**	**55**
Preserved Femoral Blood	**100**	**98**	**82**	**61**	**82**	**84**	**50**
Preserved Vitreous	**100**	**95**	**84**	**100**	**77**	**91**	**39**

Table 3: Concentrations of Morphine, Morphine-3-Glucuronide and Morphine-6-Glucurionide observed in a variety of autopsy samples.

Case	Matrix	Morphine	M-3-G	M-6-G
1	CB	0.17	0.3	0.1
	FB	0.1	0.32	0.11
	L	0.28	0.29	0.15
	M	BLOQ	0.06	BLOQ
	BM	0.08	0.14	BLOQ
	Br	0.15	BLOQ	BLOQ
	VH	0.07	0.07	0.02
2	CB	1.02	0.37	0.09
	FB	0.37	0.05	0.02
	L	2.26	1.61	0.61
	M	0.25	BLOQ	0.05
	BM	0.97	BLOQ	0.09
	Br	0.75	BLOQ	0.05
	VH	0.22	0.04	0.02
3	CB	0.1	0.29	0.13
	FB	0.06	0.12	0.04
	L	0.28	0.22	0.2
	M	0.07	0.07	0.06
	BM	0.19	0.1	0.1
	Br	0.14	0.08	0.06
	VH	0.04	0.2	0.06
4	CB	0.53	0.14	0.05
	FB	0.22	0.03	BLOQ
	L	0.95	1.68	0.57
	M	0.36	BLOQ	BLOQ
	BM	0.38	BLOQ	BLOQ
	Br	0.82	BLOQ	BLOQ
	VH	0.06	BLOQ	BLOQ
5	CB	0.41	0.38	0.14

	FB	0.25	0.09	0.03
	L	2.55	2.09	1.76
	M	0.48	0.08	ND
	BM	0.5	0.14	0.09
	Br	0.14	ND	ND
	VH	0.19	0.23	0.07
6	CB	0.15	0.13	0.04
	FB	0.07	0.02	BLOQ
	L	0.48	0.4	0.19
	M	0.27	ND	ND
	BM	0.21	ND	ND
	Br	0.12	ND	ND
	VH	0.04	0.02	BLOQ
7	CB	1.85	0.97	0.21
	FB	1.35	0.64	0.14
	L	2.21	4.68	1.67
	M	1.13	0.24	0.15
	BM	3.23	0.32	0.07
	Br	1.96	1.12	0.04
	VH	0.33	0.31	0.07
8	CB	0.3	0.71	0.15
	FB	0.21	0.27	0.06
	L	**0.78**	**1.25**	**0.46**
	M	ND	ND	ND
	BM	0.31	0.07	0.06
	Br	0.24	BLOQ	0.03
	VH	0.11	0.12	0.04

Case	Matrix	Morphine	M-3-G	M-6-G
9	CB	0.49	0.89	0.28
	FB	0.33	0.41	0.13
	L	1.12	1.04	0.43

	M	ND	0.2	0.06
	BM	0.24	0.19	0.06
	Br	0.27	0.05	BLOQ
	VH	0.18	0.46	0.1
10	CB	0.56	0.73	0.17
	FB	0.57	0.48	0.09
	L	0.69	0.53	0.19
	M	0.5	0.11	ND
	BM	0.59	0.2	ND
	Br	0.51	0.03	ND
	VH	0.1	0.12	0.02
11	CB	0.03	0.06	BLOQ
	FB	0.13	0.13	0.04
	L	0.98	2.01	0.11
	M	0.13	0.23	0.09
	BM	0.07	0.11	BLOQ
	Br	0.06	BLOQ	ND
	VH	0.03	0.15	0.03
12	CB	0.02	0.14	0.03
	FB	0.03	0.11	0.02
	L	0.08	0.09	BLOQ
	M	BLOQ	BLOQ	ND
	BM	BLOQ	BLOQ	ND
	Br	0.07	BLOQ	ND
	VH	0.03	0.05	0.02
13	CB	0.29	0.26	0.06
	FB	0.08	0.14	0.04
	L	0.21	0.25	0.15
	M	0.12	0.13	0.08
	BM	0.21	0.12	0.07
	Br	0.21	0.11	0.05

	VH	0.15	0.19	0.06
14	CB	0.2	0.27	0.09
	FB	0.1	0.05	0.02
	L	0.44	0.23	0.16
	M	0.06	BLOQ	ND
	BM	0.05	BLOQ	ND
	Br	0.18	BLOQ	ND
	VH	0.06	0.08	0.02
15	CB	BLOQ	0.01	ND
	FB	BLOQ	0.01	ND
	L	ND	ND	ND
	M	ND	ND	ND
	BM	ND	ND	ND
	Br	ND	ND	ND
	VH	BLOQ	ND	ND
16	CB	0.02	0.06	0.01
	FB	0.02	0.02	ND
	L	0.08	BLOQ	BLOQ
	M	0.06	BLOQ	ND
	BM	BLOQ	BLOQ	ND
	Br	0.07	BLOQ	ND
	VH	0.06	0.05	0.01

Case	**Matrix**	**Morphine**	**M-3-G**	**M-6-G**
17	CB	0.13	0.24	0.07
	FB	0.14	0.33	0.07
	L	0.37	0.29	0.21
	M	0.48	0.11	ND
	BM	ND	ND	ND
	Br	0.11	0.04	ND

	VH	0.07	0.09	BLOQ
18	CB	0.66	0.06	0.05
	FB	0.16	0.01	0.01
	L	0.58	0.13	0.18
	M	0.28	0.07	ND
	BM	0.23	ND	ND
	Br	0.62	ND	ND
	VH	0.16	0.02	ND
19	CB	0.26	0.43	0.1
	FB	0.19	0.17	0.05
	L	0.7	1.17	0.57
	M	0.22	BLOQ	ND
	BM	ND	BLOQ	ND
	Br	0.25	BLOQ	ND
	VH	0.08	0.15	0.01
20	CB	0.3	0.56	0.11
	FB	0.33	0.38	0.07
	L	0.73	0.17	BLOQ
	M	BLOQ	0.07	ND
	BM	BLOQ	0.06	ND
	Br	0.27	0.05	BLOQ
	VH	0.15	0.25	0.06
21	CB	0.02	0.02	ND
	FB	0.02	0.02	ND
	L	0.08	0.1	ND
	M	BLOQ	BLOQ	ND
	BM	BLOQ	BLOQ	ND
	Br	BLOQ	BLOQ	ND
	VH	0.02	0.02	ND
22	CB	0.11	0.04	ND
	FB	0.01	0.02	ND

	L	0.19	0.18	0.13
	M	0.14	BLOQ	ND
	BM	0.12	BLOQ	ND
	Br	0.08	BLOQ	ND
	VH	0.02	BLOQ	ND
23	CB	0.04	0.08	ND
	FB	0.03	0.08	ND
	L	0.13	0.08	ND
	M	0.01	ND	ND
	BM	0.1	ND	ND
	Br	0.04	ND	ND
	VH	0.02	0.06	ND
24	CB	BLOQ	0.09	ND
	FB	BLOQ	0.02	ND
	L	0.05	0.09	ND
	M	ND	ND	ND
	BM	0.01	ND	ND
	Br	0.01	ND	ND
	VH	BLOQ	ND	ND

Case	Matrix	Morphine	M-3-G	M-6-G
25	CB	0.78	1.14	0.48
	FB	0.78	1.12	0.66
	L	1.34	1.85	0.83
	M	0.64	0.23	0.09
	BM	0.49	0.04	ND
	Br	0.46	0.04	ND
	VH	0.27	0.32	0.07
26	CB	0.33	0.24	0.12
	FB	0.14	0.03	0.02

	L	0.33	0.39	0.28
	M	0.09	ND	ND
	BM	0.25	ND	ND
	Br	0.36	ND	ND
	VH	0.05	0.03	0.02
27	CB	0.01	0.03	ND
	FB	0.02	0.04	0.01
	L	ND	BLOQ	ND
	M	ND	BLOQ	ND
	BM	0.12	0.01	ND
	Br	0.04	BLOQ	ND
	VH	0.02	0.02	ND
28	CB	0.09	0.19	0.04
	FB	0.1	0.53	0.08
	L	0.47	0.24	0.09
	M	0.11	0.25	0.08
	BM	0.11	0.17	ND
	Br	0.28	0.08	ND
	VH	0.12	0.65	0.08
29	CB	0.35	0.79	0.16
	FB	0.21	0.33	0.05
	L	0.67	0.59	0.36
	M	0.19	0.07	ND
	BM	0.49	0.07	ND
	Br	0.21	0.05	ND
	VH	0.08	0.18	0.03
30	CB	0.49	1.74	0.21
	FB	0.51	0.99	0.12
	L	1.19	1.7	0.24
	M	0.89	0.91	BLOQ
	BM	0.3	0.13	BLOQ

	Br	0.5	BLOQ	BLOQ
	VH	0.19	0.33	0.03
31	CB	0.59	1.74	0.35
	FB	0.19	0.27	0.04
	L	0.88	0.61	0.36
	M	0.28	0.14	BLOQ
	BM	0.07	BLOQ	ND
	Br	0.17	0.04	BLOQ
	VH	0.1	0.24	0.03
32	CB	0.29	0.57	0.09
	FB	0.24	0.45	0.04
	L	0.77	1.12	0.6
	M	0.21	0.07	BLOQ
	BM	BLOQ	BLOQ	BLOQ
	Br	0.2	0.4	BLOQ
	VH	0.2	0.57	0.03

Case	Matrix	Morphine	M-3-G	M-6-G
33	CB	0.3	0.13	0.02
	FB	0.3	0.16	0.02
	L	0.77	0.94	0.54
	M	BLOQ	BLOQ	ND
	BM	0.49	BLOQ	BLOQ
	Br	0.28	BLOQ	ND
	VH	0.49	0.2	0.02
34	CB	0.85	0.9	0.29
	FB	0.39	0.17	0.06
	L	1.46	1.19	0.49
	M	0.31	0.14	BLOQ
	BM	0.05	BLOQ	ND

	Br	0.71	0.07	BLOQ
	VH	0.33	0.62	0.19
35	CB	0.01	0.02	BLOQ
	FB	0.16	0.89	0.16
	L	0.1	BLOQ	BLOQ
	M	BLOQ	BLOQ	BLOQ
	BM	BLOQ	BLOQ	BLOQ
	Br	BLOQ	BLOQ	BLOQ
	VH	0.03	0.02	0.01
36	CB	0.12	0.17	0.03
	FB	0.09	0.2	0.04
	L	0.2	0.17	0.09
	M	0.07	0.07	BLOQ
	BM	BLOQ	ND	ND
	Br	0.12	BLOQ	BLOQ
	VH	0.11	0.1	0.02
37	CB	0.08	0.27	0.03
	FB	0.1	0.48	0.03
	L	0.25	0.14	0.07
	M	BLOQ	BLOQ	BLOQ
	BM	BLOQ	ND	ND
	Br	0.09	0.04	BLOQ
	VH	0.1	0.14	0.02
38	CB	BLOQ	0.02	BLOQ
	FB	BLOQ	0.01	BLOQ
	L	BLOQ	BLOQ	BLOQ
	M	ND	ND	ND
	BM	ND	ND	ND
	Br	BLOQ	BLOQ	ND
	VH	0.01	0.03	BLOQ
39	CB	0.22	0.2	0.04

	FB	0.15	0.06	0.01
	L	0.44	0.79	0.24
	M	0.2	BLOQ	ND
	BM	BLOQ	ND	ND
	Br	0.06	BLOQ	ND
	VH	0.07	0.04	BLOQ
40	CB	0.19	0.71	0.13
	FB	0.17	0.4	0.06
	L	0.41	0.46	0.25
	M	0.13	0.13	BLOQ
	BM	BLOQ	BLOQ	ND
	Br	0.2	0.07	BLOQ
	VH	0.08	0.31	0.04

Case	Matrix	Morphine	M-3-G	M-6-G
41	CB	0.07	0.12	0.02
	FB	0.05	0.14	0.02
	L	0.14	0.11	0.05
	M	BLOQ	BLOQ	BLOQ
	BM	BLOQ	BLOQ	BLOQ
	Br	0.05	BLOQ	BLOQ
	VH	0.04	0.1	0.01
42	CB	0.35	0.26	0.06
	FB	0.19	0.03	BLOQ
	L	0.9	0.81	0.23
	M	0.09	BLOQ	BLOQ
	BM	0.3	BLOQ	BLOQ
	Br	0.21	BLOQ	ND
	VH	0.07	0.02	BLOQ
43	CB	0.01	0.12	0.01

	FB	BLOQ	0.05	BLOQ
	L	BLOQ	BLOQ	BLOQ
	M	BLOQ	BLOQ	BLOQ
	BM	BLOQ	BLOQ	BLOQ
	Br	BLOQ	BLOQ	BLOQ
	VH	0.03	0.05	BLOQ
44	CB	0.27	0.03	BLOQ
	FB	0.13	BLOQ	BLOQ
	L	0.56	0.84	0.29
	M	0.13	BLOQ	BLOQ
	BM	0.22	BLOQ	BLOQ
	Br	0.27	BLOQ	ND
	VH	0.13	0.07	BLOQ

Abbreviations: CB – Cardiac Blood, FB – Femoral Blood, L – Liver, M – Muscle (Psoas), BM – Bone Marrow, Br – Brain (Thalamus), VH – Vitreous Humour, ND- Not Detected, BLOQ – Detected Below Limit of Quantitation, M-3-G – Morphine-3-Glucuronide, M-6-G – Morphine-6-Glucuronide

Concentrations: Fluids – mg/L Solids – mg/kg

Table 4: Morphine, Mophine-3-glucuronide and Mophine-6-glucuronide range, mean and median from all cases analysed.

Analyte Morphine	Cardiac Blood (mg/L)	Fem Blood (mg/L)	Liver (mg/kg)	Muscle (mg/kg)	Bone M (mg/kg)	Brain (mg/kg)	F/O Fem Blood (mg/L)	Vitreous (mg/L)
Range	0.01-1.85	0.01-1.35	0.01-2.55	0.01-1.13	0.01-3.23	0.01-1.96	0.01-1.19	0.01-0.49
Mean	0.30	0.20	0.65	0.21	0.26	0.26	0.18	0.11
Median	0.21	0.14	0.48	0.13	0.12	0.18	0.14	0.08
M3G								
Range	0.01-1.74	0.01-1.12	0.01-4.68	0.01-0.91	0.01-0.32	0.01-1.12	0.01-0.88	0.01-0.65
Mean	0.38	0.23	0.71	0.09	0.06	0.07	0.20	0.16
Median	0.24	0.14	0.39	0.07	0.01	0.01	0.13	0.10
M6G								
Range	0.01-0.48	0.01-0.66	0.01-1.76	0.01-0.15	0.01-0.1	0.01-0.06	0.01-0.46	0.01-0.19
Mean	0.10	0.06	0.33	0.03	0.03	0.02	0.06	0.03
Median	0.08	0.04	0.21	0.01	0.01	0.01	0.04	0.02

Table 5: Blood concentrations of morphine, morphine-3-glucuronide and morphine-6-glucuronide in postmortem (PM) and driving under the influence of drugs (DUID) cases.

Reference	Case Type	Sample	Number of cases	[Morphine] mg/L		[M3G] mg/L		[M6G] mg/L	
				Median	Range	Median	Range	Median	Range
This Study	PM	Femoral	44	0.14	0.01 - 1.19	0.14	0.01 - 1.12	0.04	0.01 - 0.66
16	PM	Femoral	45	0.22	0.01 – 1.1	0.25	0.0036 – 2.4	0.052	0.015 – 0.58
18	PM	Femoral	?	0.11	0.01 - 0.57				
50	PM	Femoral	8	0.26	0 – 0.8	0.36	0 - 1.04	0.1	0 - 0.29
70	PM	Femoral	126	0.22	0.01 – 3.55				
71	PM	Femoral	28	0.16	0.02 – 3.30				
68	PM	Femoral	40	0.25	0.06 – 0.80	0.43	0.13 - 2.22	0.09	0.02 - 0.5
65	PM	Femoral	32	0.08	0.007 – 1.61				
6	PM	Femoral	766	0.24	0.04 – 5.5				
6	DUID	Blood	124	0.15	0.02 - 1.13				

Table 6: r^2 values describing correlation for data where zero was included.

	Cardiac Blood	Femoral Blood	Liver	Muscle	Bone Marrow	Brain	Preserved Femoral Blood
Femoral Blood	0.793						
Liver	0.632	0.511					
Muscle	0.573	0.683	0.435				
Bone Marrow	0.698	0.703	0.389	0.476			
Brain	0.872	0.737	0.440	0.553	0.765		
Preserved Femoral Blood	0.844	0.917	0.485	0.579	0.739	0.788	
Preserved Vitreous	0.448	0.446	0.427	0.219	0.244	0.344	0.419

Table 7: r^2 values describing correlation for data with cardiac blood results higher and lower than 0.3 mg/L

	Cardiac Blood	Fem Blood	Liver	Muscle	Bone Marrow	Brain	F/O Fem Blood
correlation for data with cardiac blood results higher than 0.3 mg/L							
Fem Blood	0.706						
Liver	0.489	0.330					
Muscle	0.560	0.716	0.384				
Bone Marrow	0.728	0.712	0.335	0.490			
Brain	0.860	0.660	0.292	0.520	0.766		
F/O Fem Blood	0.805	0.918	0.324	0.632	0.785	0.760	
Vitreous	0.202	0.230	0.217	0.080	0.144	0.139	0.194
correlation for data with cardiac blood results lower than 0.3 mg/L							
Fem Blood	0.571						
Liver	0.473	0.595					
Muscle	0.311	0.320	0.332				
Bone Marrow	0.049	0.003	0.042	0.004			
Brain	0.622	0.399	0.426	0.167	0.125		
F/O Fem Blood	0.438	0.541	0.257	0.095	0.003	0.265	
Vitreous	0.552	0.540	0.342	0.152	0.008	0.571	0.518